

The Stereochemistry of the Reaction Catalyzed by D-Glyceric 3-Dehydrogenase*

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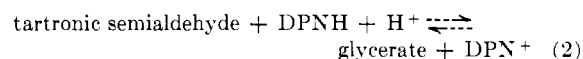
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Tritium has been used to show that the D-glyceric dehydrogenase of glycolate-adapted *Escherichia coli* causes a direct transfer of hydrogen from the β side of the reduced nicotinamide ring of diphosphopyridine nucleotide to the aldehyde carbon atom of tartronic semialdehyde. The steric position of the transferred hydrogen in the glycerate has been shown to be equivalent to that of the hydrogen atom at C-2 of L-lactate.

Three different dehydrogenases which act on glycerate have previously been characterized with respect to their stereospecificity for DPN.¹ These are: parsley leaf D-glyceric dehydrogenase, and muscle and potato tuber L-lactic dehydrogenases (Loewus and Stafford, 1960). All of these enzyme reactions have been shown to involve a transfer of hydrogen between C-2 of glycerate and the α position at C-4 of the nicotinamide ring of DPN. The present paper describes a similar study of a glyceric dehydrogenase present in extracts of glycolate-adapted *Escherichia coli*. This latter enzyme, described by Krakow and Barkulis (1956) and Krakow *et al.* (1961), operates in a metabolic reaction sequence in which the first step is an oxidation of glycolate to glyoxylate. The second step involves a carboligase-catalyzed conversion of glyoxylate to CO₂ and a compound tentatively identified as tartronic semialdehyde, as shown in equation (1). The third step—the object of our present interest—involves the enzymic reduc-



tion of the carboligase reaction-product to glycerate by DPNH. The evidence that tartronic semialdehyde is the oxidant for DPNH in this reaction, as shown in equation (2), was based largely on the identification of glycerate as the reduction product and on the demonstration that hydroxypyruvate was relatively inactive as a substrate in the enzyme reaction. This could not



be regarded as complete evidence for the identifica-

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¹ The abbreviations used are: DPN, diphosphopyridine nucleotide; DPN⁺, the oxidized form of DPN; DPN(T)⁺, DPN⁺ containing tritium at the para (or 4) position of the nicotinamide ring; DPNH, reduced DPN; DPNT, DPNH containing tritium at the reduced (4) position of the pyridine ring. The prefixes α and β are used to indicate the position of the tritium on the methylene group in the reduced nicotinamide ring. The α position is the one to which hydrogen is transferred by the yeast alcohol dehydrogenase reaction. Thus, when DPN(T)⁺ is reduced by ethanol in the presence of yeast alcohol dehydrogenase, the product is β -DPNT.

tion of tartronic semialdehyde, since an enol common to hydroxypyruvate and tartronic semialdehyde could also yield glycerate on reduction.

The present experiments were undertaken with the double purpose of identifying the oxidized form of the substrate of reaction (2) and of determining the stereospecificity for DPN of this glyceric dehydrogenase reaction, for comparison with available information on other glyceric dehydrogenases. These aims were achieved by the use of tritium as a tracer. Evidence will be presented that, in the course of reaction (2), tritium is transferred from β -DPNT to C-3 of tartronic semialdehyde. The steric position of the transferred hydrogen in the glycerate has also been determined.

Since the present study was completed, Gotto and Kornberg (1961a,b) have described the isolation in crystalline form of an enzyme which they term tartronic semialdehyde reductase. This enzyme, obtained from glycolate-adapted *Pseudomonas ovalis* chester, appears to catalyze the same reaction as the *E. coli* enzyme employed in the present studies. In an extensive study of the metabolism of C-2 compounds in microorganisms, Kornberg and Gotto (1961) have also described the carboligase reaction.

RESULTS AND DISCUSSION

The Oxidation of β -DPNT.—Extracts of glycolate-adapted *E. coli* contain the carboligase together with the glyceric dehydrogenase. All experiments were performed with a protein fraction containing both enzymes. The carboligase reaction of equation (1) is essentially irreversible and goes to completion. The course of the reaction may be followed by microtitration of the hydrogen ions consumed in an unbuffered reaction mixture (Schwartz and Meyers, 1958). Aliquots of such a solution were used immediately after the carboligase reaction was complete, to effect the reoxidation of specifically labeled reduced diphosphopyridine nucleotide. The equilibrium point of reaction (2) favors the oxidation of DPNH. It had previously been shown that the amount of pyridine nucleotide oxidized under such circumstances was equivalent on a molar basis to half the amount of glyoxylate originally employed, and

that an equivalent amount of glycerate was formed (Krakow and Barkulis, 1956; Krakow *et al.*, 1961). The first experiments with labeled pyridine nucleotide showed that the reaction in question involved removal of hydrogen from the β rather than the α position of the reduced nicotinamide ring of DPN, and that the hydrogen so removed did not appear in the water of the reaction medium. For these experiments, β -DPNT was prepared by reducing DPN(T)⁺ with ethanol in the presence of yeast alcohol dehydrogenase. The specific activity of the nicotinamide of each sample of β -DPNT was determined after enzymatic reoxidation by acetaldehyde. This was necessary because the labeled pyridine nucleotide was diluted by addition of unlabeled carrier both before and after reduction, and these dilutions, which were made by weight, were not accurate. Enzymatic reoxidation of β -DPND by acetaldehyde has previously been shown to involve no removal of label. The radioactivity of the nicotinamide after the oxidation of the β -DPNT by acetaldehyde therefore represents the radioactivity actually added in the β -DPNT. Table I contains the results of two separate repre-

TABLE I
THE OXIDATION OF β -DPNT WITH *E. coli* GLYCERIC DEHYDROGENASE

Exper. No.	cpm/ μ mole Nicotinamide After Reoxidation of β -DPNT	
	By Acetaldehyde and Yeast Alcohol Dehydrogenase	By Tartronic Semialdehyde and <i>E. coli</i> GlycERIC Dehydrogenase
1	33,000	4,160
2	29,300	2,830

sentative experiments. Oxidation of the β -DPNT by tartronic semialdehyde resulted in the removal of 87% of the tritium in experiment 1, and of 90% of the tritium in experiment 2. Samples of water were also recovered from the reaction mixtures and counted. In experiment 1, 1080 cpm per μ mole β -DPNT oxidized by *E. coli* enzyme was recovered in the water. In experiment 2, 890 cpm per μ mole was recovered. The conclusion was drawn that most of the tritium removed from the reduced position of β -DPNT must have been transferred directly to the glycerate. This conclusion was confirmed by isolating and counting the glycerate (Table II).

TABLE II
LOCATION OF TRITIUM IN GLYCERIC ACID
The glycolic acid was obtained by permanganate oxidation of the glycerate, and the glyoxylate was obtained by periodate oxidation.

Exper. No.	cpm/ μ mole	
	Glycolic Acid (T at C-3 of Glycerate)	Glyoxylic Acid (T at C-2 of Glycerate)
1	34,000	32,300
2	29,300	29,800

The appearance of a small amount of tritium in the water in the experiments of Table I may be explained by the occurrence of a small proportion of unidentified side-reactions in the unpurified

enzyme preparations used in these experiments. The direct transfer of most of the isotope to glycerate made it appear improbable that an enol was the direct precursor of glycerate, since reduction of an enol might be expected to occur by way of a flavoprotein, without demonstrable hydrogen transfer between pyridine nucleotide and glycerate. If, on the other hand, a ketone or aldehyde is the precursor of glycerate, it would be reasonable (though perhaps not certain) to anticipate a direct transfer of a hydrogen atom from reduced pyridine nucleotide to the carbonyl carbon atom (Vennesland, 1958).

The retention of 10 to 15% of the tritium after oxidation of the β -DPNT by the bacterial enzyme cannot be regarded as evidence that the stereospecificity of the *E. coli* glyceric dehydrogenase is not complete. Similar isotope retention was also observed when the same β -DPNT was oxidized with other enzymes, including glutamic dehydrogenase. When the latter enzyme was previously employed for oxidation of β -DPND, the D was removed more completely. The DPN(T)⁺ used in the present experiments has been shown to contain no nonexchangeable label except at position 4 of the nicotinamide ring. The relatively high retention of tritium as compared to deuterium should probably be ascribed to a larger isotope discrimination effect with tritium. Such an effect could operate indirectly in the following way. If the oxidation of DPNH occurs more rapidly than the oxidation of β -DPNT, then, even though the enzyme reaction is run as nearly as possible to completion, a small amount of highly labeled β -DPNT will remain in the reaction mixture. This highly labeled β -DPNT will to some extent be oxidized nonenzymatically during the subsequent manipulations carried out for the isolation of the nicotinamide. The nonenzymatic oxidation will involve mainly retention of tritium, and this tritium will appear in the isolated nicotinamide. Percentage-wise it will appear to represent a relatively large proportion of the total DPN, because of the high concentration of isotope which may be present in a relatively small amount of residual DPNT.

The Location of Tritium in Glyceric Acid.—If tartronic semialdehyde is the precursor of glycerate, the tritium transferred in the enzyme reaction should appear at C-3, whereas the reduction of hydroxypyruvate should lead to the formation of C-2 labeled glycerate. Three different procedures were employed to locate the tritium in the glycerate. The first involved periodate oxidation by the procedure of Bassham *et al.* (1950) as modified by Aronoff (1951). In this method, C-3 of glycerate is converted to formaldehyde and the two-carbon fragment is converted to glyoxylate. The formaldehyde was recovered as the formyl-dimeton derivative (Aronoff and Vernon, 1950), and the glyoxylate was separated by paper chromatography. The latter compound contained no detectable tritium (Table II), whereas the formyl-dimeton derivative was radioactive. However, only about 40 to 50%

radioactivity of the glycerate was recovered, showing that some loss had occurred, possibly in a side-reaction during the periodate oxidation.

Another degradation procedure involved oxidation of glycerate to CO_2 and glycolate with KMnO_4 at neutral pH. The yield of glycolate, which was separated by chromatography, was low, but sufficient for adequate counting. The radioactivity in the glycolate accounted for all of the label in the glycerate, as shown in Table II. Since all of the hydrogen in the glycolate is derived from that attached to C-3 of glycerate, these results showed that all of the tritium transferred to glycerate from β -DPNT was located on C-3.

Further confirmation of this conclusion was obtained by converting the glycerate enzymically to phosphoglycerate (Hanson and Hayashi, 1962), reducing the latter enzymically to triose phosphate, and treating the latter with triose-phosphate isomerase. Any tritium which was located at C-2 of glycerate should have exchanged into the water of the medium under these circumstances. There was no detectable tritium found in the water. This confirmed the previous conclusion that all of the tritium was located at C-3. The activity of the glycerate in the enzyme reaction sequence employed also showed that it is D-glycerate that is formed when tartronic semialdehyde is reduced by DPNH in the presence of the *E. coli* dehydrogenase preparation.

On the basis of the evidence presented, the *E. coli* glyceric dehydrogenase has been termed D-glyceric 3-dehydrogenase. (The glyceric dehydrogenase of leaves which catalyzes the oxidation of glycerate to hydroxypyruvate [Loewus and Stafford, 1960] may be termed D-glyceric 2-dehydrogenase.) The crude *E. coli* extracts employed catalyze the oxidation of TPNH almost as well as that of DPNH by the end-product of the carboligase reaction. The crystalline tartronic semialdehyde reductase described by Gotto and Kornberg (1961a,b) has likewise been reported to be active with both pyridine nucleotides.

It is of interest that the *E. coli* glyceric dehydrogenase has β -stereospecificity for DPN whereas all other glyceric dehydrogenases hitherto examined have α -stereospecificity. This difference can be associated with the fact that the *E. coli* glyceric dehydrogenase oxidizes glycerate at the C-3 position whereas the other glyceric dehydrogenases studied all involve removal of hydrogen from the C-2 or carbinol carbon atom adjacent to the carboxyl group. The generalization has been made that the pyridine nucleotide dehydrogenases which involve the oxidation of α -hydroxy carboxylic acids at the α or C-2 position all have α -stereospecificity for the hydrogen transfer to the pyridine nucleotide (Vennesland, 1961). Since the *E. coli* glyceric dehydrogenase operates at the C-3 position, the β -stereospecificity of this particular reaction does not invalidate the generalization.

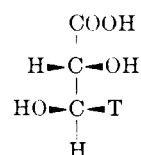
The Stereospecificity of the Hydrogen Transfer for C-3 of Glycerate.—There are two hydrogen atoms attached to C-3 of glycerate, and no racemization

or inversion of these hydrogen atoms would be expected during oxidation of the glycerate to glycolate. Rose (1958) has shown that the glycolic acid oxidase of tobacco leaf (Zelitch, 1955) catalyzes the removal of tritium from the position which is sterically equivalent to that of the hydrogen atom attached to C-2 of L-lactate. This provided a procedure for locating the steric position of the tritium in the labeled glycerate formed on enzymic reduction of tartronic semialdehyde with β -DPNT. The glycolate derived from the tritium-labeled glycerate was oxidized by glycolic oxidase, and most of the label was shown to be lost to the water. Table III shows the results obtained in

TABLE III
OXIDATION OF GLYCOLIC ACID BY GLYCOLIC ACID OXIDASE

Exper. No.	O_2 Consumed μmoles	Tritium Recovered	
		In H_2O (%)	In Residue (%)
1	3.5	87	0
2	3.7	94	0

two experiments in which 3.7 μmoles of labeled glycolate were oxidized with glycolic oxidase from spinach (Zelitch and Ochoa, 1953). A crystalline preparation of tobacco leaf glycolic oxidase, which was generously provided by Dr. Zelitch, gave similar results. The spinach enzyme has the same stereospecificity as the glycolic oxidase from tobacco leaves. On the basis of the evidence summarized, one may write the projection formula of the tritiated glycerate formed on enzymatic transfer of T from DPN to the aldehyde carbon atom of tartronic semialdehyde as follows (in the terminology of Cahn *et al.* (1956), the configuration of C-3 would be R).



EXPERIMENTAL

Materials.—Calcium DL-glycerate, obtained from Aldrich Chemical Company, was recrystallized from hot alcohol and water. Catalase, DPN, and sodium glyoxylate monohydrate were purchased from Sigma Chemical Company. Yeast alcohol dehydrogenase, yeast 3-phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase from skeletal muscle (rabbit), and triose isomerase were obtained from California Corporation for Biochemical Research.

Preparation of *E. coli* Enzyme Extract.—*Escherichia coli*, Crook's strain, was grown in a medium containing glycolate as the sole carbon source (Krakow and Barkulis, 1956; Krakow *et al.*, 1961). After 18 to 24 hours of growth with aeration, the bacteria were harvested in a continuous-flow centrifuge, washed with cold distilled water, and recovered by centrifugation. The well-packed

mass of cells was ground in a mortar with Alumina A-303 (Alcoa) and the extract was fractionated, as described by Hanson and Hayashi (1962). The protein which precipitated between 24.8 and 50.6% saturation with $(\text{NH}_4)_2\text{SO}_4$ (at 0°) contained both the carboligase and the glyceric dehydrogenase activity and was used in all experiments here described. It was dissolved by addition of 10^{-3} M potassium phosphate buffer of pH 7.0, and dialyzed overnight against the same buffer immediately before use. The undialyzed enzyme solution could be stored in the frozen state, but storage for more than 3 days was avoided because of the relatively large loss of glyceric dehydrogenase activity.

Pyridine Nucleotide.— β -DPNT was prepared from DPN(T)⁺ by reduction with ethanol in the presence of yeast alcohol dehydrogenase and isolated by precipitation with ethanol as described by Loewus *et al.* (1953). The DPN(T)⁺ was prepared by Julio Ludowieg (Ludowieg and Vennesland, unpublished). The hydrolysis of DPN⁺ to nicotinamide by heating in alkaline phosphate buffer, and the isolation and purification of the nicotinamide, were carried out as previously described (Loewus *et al.*, 1953; Marcus *et al.*, 1958).

The Oxidation of β -DPNT by the Bacterial Enzyme System.—For the generation of tartronic semialdehyde, 30 μ moles of sodium glyoxylate, 10 μ moles of MgCl_2 , 100 μg of thiamine pyrophosphate, and 0.4 ml of *E. coli* enzyme extract were added to sufficient water to bring the final volume to about 5.0 ml, and the pH was maintained at 7.0 by the gradual addition of 15 μ moles of HCl (Schwartz and Meyers, 1958). For the experiments described in Table I, 0.3 ml of the above reaction mixture was added to a solution containing 0.3 to 0.4 μ moles of β -DPNT, 100 μ moles of tris(hydroxymethyl)aminomethane-HCl buffer of pH 7.4, and water to bring the final volume to 3.0 ml. The reaction was carried out in a Beckman cuvet so that the oxidation of the reduced DPN could be followed spectrophotometrically. The reaction with alcohol dehydrogenase was set up similarly with an identical amount of β -DPNT, but 10 μ moles of freshly distilled acetaldehyde and 0.15 mg of yeast alcohol dehydrogenase were substituted for the bacterial enzyme system. After completion of the oxidation of β -DPNT (which was rapid), the enzymes were heat inactivated. Samples of water were recovered by lyophilization, and the oxidized DPN was hydrolyzed to nicotinamide, which was isolated by ether extraction after addition of 50 mg unlabeled nicotinamide as carrier. For the experiments of Table II, the procedures were similar, except that about threefold larger amounts of bacterial enzyme system and β -DPNT were employed.

Isolation and Degradation of Glycerate.—After oxidation of β -DPNT by the carboligase reaction product, the proteins were precipitated by heating and removed by centrifugation. The tritiated glycerate in the supernatant was diluted with 50

to 100 mg of unlabeled calcium glycerate. The mixture was heated briefly and filtered, and hot alcohol was added until the solution was faintly turbid. After overnight refrigeration, the calcium glycerate was collected on a Buchner funnel. It was recrystallized from hot water and alcohol, and dried *in vacuo*.

Periodate oxidation of a sample of the glycerate was carried out as described by Aronoff (1951). The reaction vessel was covered to prevent exposure to light. The glyoxylic acid formed was recovered by chromatographing the reaction mixture on #3 Whatman filter paper. The upper layer of a 1:1 mixture of isoamyl alcohol-5 M formic acid was used as solvent. The glyoxylic acid band was identified by comparison with a standard sample which was chromatographed simultaneously. The acids were visualized by spraying with brom-cresol green acid-base indicator.

Approximately 3 ml of eluate containing glyoxylic acid was cooled to 0° and brought to pH 5.5 by careful addition of 0.1 N NaOH. Acetone was added to the solution until it became turbid. After overnight refrigeration, the precipitate of sodium glyoxylate was collected and reprecipitated from water at 40° and acetone. The crystalline sodium glyoxylate monohydrate was recovered after overnight refrigeration.

The oxidation of glyceric acid to glycolic acid and CO_2 was carried out with neutral KMnO_4 . Fifty mg of calcium glycerate and 50 mg of KMnO_4 were dissolved in 3 ml of water and allowed to stand at room temperature for about 3 hours. The precipitate which formed was removed by filtration and washed with water. The washings were combined with the filtrate, and the solution was passed through a small column of Amberlite IRC-50, hydrogen form. The column was washed thoroughly with water, and the combined effluents, amounting to 10 to 20 ml volume, were concentrated to a syrup under reduced pressure. The syrup was spotted on a sheet of Whatman #3 filter paper, together with standard samples of glyceric and glycolic acid, and chromatographed in the solvent described by Buch *et al.* (1952). The acids were detected by spraying the dried chromatogram with acid-base indicator. The glycolic acid band was shredded and extracted several times with water. The combined extracts were concentrated *in vacuo* and neutralized with calcium hydroxide or sodium hydroxide. The amount of glycolate present was determined colorimetrically by the method of Calkins (1953). About 1.5 to 3 mg of sodium glycolate was obtained. Aliquots of the glycolate solution were counted directly or carrier calcium glycolate was added so that a sample of solid calcium glycolate could be obtained by addition of ethanol to the aqueous solution.

Oxidation of Glycolate with Glycolic Oxidase.—As pointed out by Richardson and Tolbert (1961), glycolic oxidase can oxidize glyoxylate to oxalate. This latter reaction must be avoided if reasonable yield of glyoxylate is to be obtained. In the present

experiments (Table III), the oxidation of glycolate was carried out in Warburg manometer vessels at 30°. The reaction mixtures contained 100 μ moles of tris(hydroxymethyl)aminomethane-HCl buffer of pH 7.8, 0.1 μ moles of flavin mononucleotide, 30 μ moles of sodium oxalate, 0.5 mg of crystalline catalase, 3.67 μ moles of sodium glycolate, and 0.2 ml of spinach glycolic oxidase prepared according to Zelitch and Ochoa (1953), in a volume of 1.8 ml. The oxygen consumption, followed manometrically, was complete in 20 minutes. In the presence of the buffer and added oxalate, the theoretical amount of O₂ was consumed, but only after the labeled glycolate had been rechromatographed on washed paper. Otherwise, the amount of O₂ consumed was higher than theory predicted. (The paper was washed in the developing solvent, dried, washed with water, and dried again.) Even when the theoretical amount of O₂ was consumed, the amount of glyoxylate formed (as determined by the Friedemann-Haugen method [1943]) was lower than expected by 30 to 40%. The glycolate used in the experiments of Table III had been diluted to a specific activity of about 30 cpm per μ mole. It was not practicable, at this low counting level, to isolate and count the glyoxylate formed. However, almost all the tritium added in the glycolate could be shown to be present in the water recovered by lyophilization, and the residue contained no detectable count. There was sufficient glyoxylate in the residue to permit the conclusion that the label had been stereospecifically removed in the conversion of glycolate to glyoxylate.

Determination of Tritium.—Counting was done with a Tri-Carb Liquid Scintillation Spectrometer, Model 314-DC (Packard Instrument Company). The scintillation mixture described by Bray (1960) was used for counting the organic acids. Clear solutions were obtained when 3 mg or less of the calcium salts were dissolved in 1 ml water and added to 10 ml of scintillation mixture. Other compounds were counted with a scintillation mixture containing 6.43 g of 2,5-diphenyloxazole and 107 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene (Packard Instrument Company) in one liter of Fisher certified toluene. Each weighed sample was dissolved in 0.6 ml water, to which 6 ml ethanol and 8.4 ml of scintillation mixture were added. Standards prepared from tritiated nicotinamide and samples

of tritiated water were counted in both scintillation mixtures and appropriate corrections were applied for differences in counting efficiency. All results represent averages of counts from at least two different samples prepared with different quantities of unknown to ensure against deviations from linearity between the counting rates and the amount of material counted. Except for the data in Table III, where the counting error was about 10 to 15%, all counts were made under conditions to ensure a counting error of less than 5%, and corrected for background. When unlabeled carriers were added, the results were corrected appropriately for dilution.

REFERENCES

- Aronoff, S. (1951), *Arch. Biochem. Biophys.* **32**, 237.
Aronoff, S., and Vernon, L. (1950), *Arch. Biochem.* **28**, 424.
Bassham, J. A., Benson, A. A., and Calvin, M. (1950), *J. Biol. Chem.* **185**, 781.
Bray, G. A. (1960), *Anal. Biochem.* **1**, 279.
Buch, M. L., Montgomery, R., and Porter, W. L. (1952), *Anal. Chem.* **24**, 489.
Cahn, R. S., Ingold, C. K., and Prelog, V. (1956), *Experientia* **12**, 81.
Calkins, V. (1953), *Ind. Eng. Chem. Anal. Ed.*, **15**, 762.
Friedemann, T. E., and Haugen, G. E. (1943), *J. Biol. Chem.* **147**, 415.
Gotto, A. M., and Kornberg, H. L. (1961a), *Biochim. et Biophys. Acta* **48**, 604.
Gotto, A. M., and Kornberg, H. L. (1961b), *Biochem. J.* **79**, 9p.
Hanson, R., and Hayashi, J. A. (1962), *J. Bacteriol.*, in press.
Kornberg, H. L., and Gotto, A. M. (1961), *Biochem. J.* **78**, 69.
Krakow, G., and Barkulis, S. S. (1956), *Biochim. et Biophys. Acta* **21**, 593.
Krakow, G., Barkulis, S. S., and Hayashi, J. A. (1961), *J. Bacteriol.* **81**, 509.
Loewus, F. A., and Stafford, H. A. (1960), *J. Biol. Chem.* **235**, 3317.
Loewus, F. A., Westheimer, F. H., and Vennesland, B. (1953), *J. Am. Chem. Soc.* **75**, 5018.
Marcus, A., Vennesland, B., and Stern, J. R. (1958), *J. Biol. Chem.* **233**, 722.
Richardson, K. E., and Tolbert, W. E. (1961), *J. Biol. Chem.* **236**, 1280.
Rose, I. A. (1958), *J. Am. Chem. Soc.* **80**, 5835.
Schwartz, M., and Meyers, T. C. (1958), *Anal. Chem.* **30**, 1150.
Vennesland, B. (1958), *Fed. Proc.* **17**, 1150.
Vennesland, B. (1961), Transactions of the 5th International Biochemical Congress, Moscow, in press.
Zelitch, I. (1955), *J. Biol. Chem.* **216**, 553.
Zelitch, I., and Ochoa, S. (1953), *J. Biol. Chem.* **201**, 707.